# EVALUATION OF *IN VITRO* AND *IN SILICO* ANTIMICROBIAL AND SYNERGISTIC POTENTIAL OF *ACORUS CALAMUS* COLLECTED FROM HIMACHAL PRADESH, INDIA

**SYNOPSIS**

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## INTRODUCTION

Ayurveda is one of the oldest natural systems of medicine increasing its popularity day by day as it respond to cure many chronic diseases (Kumar et al., 2017). Ayurveda becomes in its existence in India around 6,000 years ago and is the traditional healing system to maintain health. The term Ayurveda is come out by the addition of two different words “Ayu” and “Veda”. Ayustands for “Life” and Veda stands for “knowledge or science” as Ayurveda is the Science of Life. Ayurveda also preserve good health and lifespan rather than to treat and prevent from different disease (Yadav et al., 2019). Medicinal plants have been used in the preparation of medicine since ancient times and may even be considered the origin of modern medicine. According to WHO the traditional medicine can be considered as “the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to various cultures, whether explicable or not, used in the health protection, as well as in the prevention of mankind from different pathogenic microbes and in the diagnosis, improvement or treatment of physical and mental illnesses”. At present, the medicinal plants cultivated worldwide on large scale and also serve as important source to obtain the compounds which are useful for manufacturing of drugs(Salmerón-Manzano et al., 2020).

*Acoruscalamus*(Linn.) is one such a medicinal herb that found as a member of family Acoraceae and generally called as sweet flag. The term ‘acorus’ was came from the Greek word ‘acoron’, which was used by Dioscorides (Greek Physician, Pharmacologist, Botanist) and taken from the word ‘coreon’ meaning ‘pupil’ (of an eye), as it was used to remedy the diseases of eye (Khwairakpam et al., 2018).The family of the Acoraceae encompasses about 110 genera and more than 1800 species. The genus *Acorus* comprises of about forty species, however, only few species like*Acoruscalamus*(Linn.), *Acoruschristophii*, *Acorustatarinowii*(Schott.) and *Acorusgramineus* (Sol.) (Rajput et al., 2014). It is a traditional herb used from the old time to cure the variety of immune disorders, including kidney, respiratory, metabolic, neurological, gastrointestinal, and liver relateddisorders and other health disorders (Sharma et al., 2020). Sweet flag commonly found in temperate and sub-temperate regions of the world and is native to India, China, Japan, Southeast Asia, Kazakhstan, Mongolia, and Sulawesi (Khwairakpam et al., 2018).It is commonly recorded throughout India, in marshes, wild or cultivated, ascending to about 2200 m of altitude in Himalayas especiallyKarnataka, Assam, Tamil Nadu, Kerala and Andhra Pradesh(Kareem et al., 2012).Plant is commonly found in

marshy land habitat,monocotyledonous and perennial plant with branching rhizomes. The well growth habitats for sweet flag include edges of small lakes, rivers, marshes, ponds in temperature ranging from 10 to 38 °C (Khwairakpam et al., 2017). The rhizomes of *Acoruscalamus* are brownish in color from the exterior and pink in color from inner side, cylindrical in shape, curved, and branched. The plant's leaves are erect, green and sword shapedstructure which is thicker appearance in middle along with curvy margins (Sharma et al., 2020).

The prevalence of microbial infections has increased adequately in the past decades. For the treatment of various infections there is a continual demand of antimicrobial drugs but this development has led to the emergence of resistance among the various microbial strains Multidrug resistance phenomenon (MDR) is explained as insensitivity or resistance of a microbes against the administered antimicrobial medicines even though the previous sensitivity to the same medicine. According to WHO, these resistant microorganisms are able to fight against attack by antimicrobial drugs, which ultimately leads to the spreading of infections. MDR is an unavoidable natural phenomenon, posing a serious Global threat to public health (Tanwar et al., 2014).

World Health Organization in February 2017 published the list of most antibiotic-resistant pathogenic strains that posing a serious global threat to public health. The most resistance microbial pathogens are carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant and extended spectrum betalactamase (ESBL) producing Enterobacteriaceae and vancomycin-resistant *Enterococcus faecium,* methicillin-resistant *Staphylococcus aureus*, clarithromycin-resistant *Helicobacter pylori,*fluoroquinolone-resistant *Campylobacter spp*., fluoroquinolone resistance in *Salmonellae* and cephalosporin and fluoroquinolone resistance in *Neisseria gonorrhoeae*. Consequently, there is a desperate requirement for the novel antibiotics for combating against these rapidly evolving pathogens (Subramani et al., 2017). The major mechanisms involves in multidrug resistance includes efflux pumps, differential mutation or recombination rate, genetic linkage and multidrug therapy etc. The mechanisms behind the rapid increase in MDR strains include associated linkage selection, positive epistasis, niche differentiation bystander selection, and importation of MDR from a high-use population, followed by spread in a "recipient" population(Chang et al., 2015).

Molecular docking plays important role in the designing, development and discovery of drug. The method is rapidly gaining popularity, implementation and appreciation these days and based upon *In-silico*–chemico biological approach by the use of computer aided techniques (Chaudhary et al., 2016). This method simulates the molecular interaction and helpful in predicting the binding mode and affinity between receptors and ligands (Fan et al., 2019). Molecular docking is an inexpensive, safe and easy to use tool that helps the researchers in investigating, interpreting the data, explaining and determination of molecular properties using 3D structures of the compounds (Mani et al., 2016). Molecular docking is defined as bioinformatics modeling which would explain the ‘‘best-fit’’ orientation of a ligand molecule that involves the interaction with specific protein of interest and is used to analyzed the structure of the intermolecular complex formed be-tween two or more molecules (Vijesh et al., 2013).Plants contain complex variety of phytocompounds, are likely to hold at least part of the answer to our ever-increasing problem of multidrug resistant microbes (Abscal et al., 2013).

Efforts will be made in the present work to investigate the comparative effectiveness of the plant extracts of *Acoruscalamus*for their antimicrobial activity collected from the Himachal Pradesh, India. Research will be focussed to examine the synergistic effect of medicinal plant extracts and antibiotics on pathogenic microbial strains and that will help to broaden the utility of this plant material especially for successful treatment of several diseases.*In silico* studies will also perform to validate the identified bioactive compounds of *Acoruscalamus*with antimicrobial protein targets. Keeping in view the importance of *Acoruscalamus* in medical sciences, the present study entitled as “Evaluation of *In vitro* and *Insilico* antimicrobial and synergistic potential of *Acoruscalamus* collected from Himachal Pradesh, India”.

## LITERATURE REVIEW

Approximately billions dollar is invested to develop a drug by a pharmaceutical company. But it has been observed that majority of the population in world cannot afford these therapies due to high cost and safety issues. But safety is a major issue so it is necessary to identify a safe, affordable and effective drug is a challenge to modern medicine today (Yadav et al., 2019). From the last few decades most of the population again shown a notable interest in the medicinal plants. Now a daysherbal medicines and natural products are in significant demand today in the entire world. The reason behind is the increasing awareness about the limitations of the synthetic chemotherapeutic agents

(Gagan et al., 2015). According to WHO about 80% of the world population fulfill their primary health care needs by the use of traditional medicine only. Medicinal plants contain the majority of secondary metabolites and are important sources of development of curative drugs and are used to treat the various ailments (Chandera et al., 2017).

Vacha is another important medicinal herbs used in Ayurveda traditional medicine to treat different ailments and maintain health condition. *Acoruscalamus*(Linn.) is one such a medicinal herb that found as a member of family Acoraceae and most commonly named as 'sweet flag'. The term 'acorus' was came from the Greek word 'acoron', which was used by Dioscorides (Greek Physician, Pharmacologist, Botanist) and taken from the word 'coreon' meaning 'pupil' (of an eye), as it was used to remedy the diseases of eye (Khwairakpam et al., 2018).The genus *Acorus*belongs to family Acoraceae and generally comprises of about 110 genera and 1800 species. The genus *Acorus* contains about 40 species, however, only few species like*Acoruscalamus*(Linn.), *Acoruschristophii*, *Acorustatarinowii*(Schott.) and *Acorusgramineus* (Sol.) (Rajput et al., 2014). It is a traditional Indian medicinal herb which is used from the old time to cure the broad range of health disorders, including neurological, gastrointestinal, respiratory, metabolic, kidney, and liver disorders and other health disorders (Sharma et al., 2020).*Acoruscalamus* distributed throughout the tropics and subtropics areas, especially in India and Sri Lanka. It is commonly cultivated in swampland, ascending the Himalayas up to 1800 m in Sikkim.It is found abundantly in marshy areas of Manipur, Naga Hills, Kashmir and Sirmoor. Plant is regularly grown in Koratageretaluk in Karnataka state. Light alluvial soil of river bank and clayey loams are the best for growth of plant. Widely found on the margins of pounds and rivers in most English countries (Chandera et al., 2017).

Traditionally *Acoruscalamus*regarded as a "rejuvenator" for the brain and nervous system and as a best remedy for treatment of digestive disorders. The rhizome part of *Acoruscalamus* is used mainly for curing appetite, fever, stomach cramps, tooth ache and cholic(Chandera et al., 2017).*Acoruscalamus* (Vacha)is very important treatment of the various types of the diseases like epilepsy, mental ailments, chronic diarrhea, dysentery, bronchial catarrh, intermittent and tumors. It also has the insecticidal, tranquilizing, antidiarrhoeal antifungal, antibacterial, antioxidant, anticholinesterase antidyslipidemic, neuroprotective, spasmolytic, vascular modulator activities. Sweet flag also valuable in preparation of some beneficial herbal formulations like *Vachadichurna, Vachadighrita, Vachavaleha*etc(Gagan et al., 2015).The medicinal significance of the Acoraceae is

constantly increasing due to presence of various bioactive compounds having beneficial pharmacological applications such as, anti-inflammatory/ immunosuppressive, anti-adipogenic, antimicrobial, fungicidal, insulin sensitizing/antidiabetic, neuroprotective activities (Sabhita et al., 2014). *Acoruscalamus*has tremendous potency in treatment of wide variety of ailments and also indicated as brain tonic as helpful in improvement of memory and intellect.Asarone, beta-asarone, eugenol, methyl eugenol, tannins are the major constituents present which has the wide range of pharmacological applications. Traditionally used in curing various diseases like diarrhea, slurred speech, piles, indigestion, acid gastritis, headache,edema, skin diseases, eye diseases, colic, heart disease and ear diseases (Rashmi et al., 2017).

***Acoruscalamus***

**Figure 1: Figure showing the *Acoruscalamus***

| **Table 1: Table showing the Taxonomic Classification of *Acoruscalamus*(Imami et al., 2013)** | |
|---|---|
| **Kingdom** | Plantae |
| **Subkingdom** | Tracheobionta |
| **Super division** | Spermatophyta |
| **Division** | Magnoliophyta |
| **Class** | Liliopsida |
| **Subclass** | Arecida |
| **Order** | Arales |
| **Family** | Acoraceae |
| **Genus** | *Acorus* |
| **Species** | *calamus*Linn., *griffithii*Schott.,*belangeii*Schott. |

**Vernacular name**

| **Table 2: Table showing vernacular names of *Acoruscalamus*(Umamaheshwari et al., 2018)** | |
|---|---|
| **Languages** | **Local Name** |
| **Arabic** | Vaj, Vash, OudulVaj |
| **Sanskrit** | Bhadra, BhutanashiniVacha |
| **Arabic Hindi** | Bach, Ghorbach, Safedbach |
| **Gujarati** | Gandhilovaj, Godavaj |
| **Kashmir** | Vachi, Vaigandar |
| **Persian** | Agar, Agarturki |
| **Kannada** | Baje, Vasa |
| **English** | Sweetflag, Acoruscalamus, Myrtle grass |
| **Urdu** | Bach, Vaj |
| **Tamil** | Vashambu, pullaivalathi |
| **Nepali** | Bojho |
| **Ayurvedic** | Vacha |
| **Unani** | Vajturki, Bacch |

**Distribution**

Sweet flag commonly found in temperate and sub-temperate regions of the world and is indegenous to India, Asia, Mongolia, China, Japan, South East,Kazakhstan, and Sulawesi (Khwairakpam et al., 2017).This herb naturally recorded all over India like in swamp areas and also cultivated up to an altitude of 2200 m in the Himalayas especially Karnataka, Assam, Tamil Nadu, Kerala and Andhra Pradesh(Kareem et al., 2012). In India *Acoruscalamus* is cultivated in the swampy areas of Kashmir, Shirmaur, Manipur, Nagahills, Koratageretaluka and Karnataka(Gagan et al., 2015).

**Botanical Description**

'Sweet flag'is a perennial herb and usually grow in marshy areas, monocotyledon, 1-4 feet tall in height and belongs to the family Acoraceae. The morphological structure of this herb generally consists of tufts of the basal roots that directly come out from the spreading rootstock. This herb consists of a branched rhizome part which is a root like subterranean stem, always growth in horizontal direction from which at the specific

interval tuft of basal leaves grows above ground in a population and fine coarse fibrous roots develops below (Gagan et al., 2015).

**Morphological character**

The morphological characters aregreen and sword leaves, creeping roots, cylindrical, long and branched rhizome.

**Rhizome**

The branching rhizomes parts of the plant are root like stems that grows in horizontal direction under the ground. Rhizome of this plant is long, indefinite branched, smooth, cylindrical, pale green in colorand thick up to 2.5 cm. Tufts of basal leaves grows above ground and present at intervals along these rhizomes while coarse fibrous roots grow below. The plant propagates by its rhizomes. Internally the rhizome is whitish pink in color and crushed forms have pleasant aromatic fragrance although it has a bitter taste.

**Figure 2: Figure showing the rhizome part of *Acoruscalamus***

**Roots**

The underground part of plant consistsof long creeping roots along with the root fibers which grows out just below the surface of the soil. The thickness of roots is about 1 cm and helps the plant in absorption of water and dissolved minerals for growth(Kumar et al., 2013).

**Figure 3: Figure showing the Roots of *Acoruscalamus***

**Leaves**

The leaves of the plant arise from the spreading rhizome are erect, green and sword in shaped, resembling in appearance to Iris leaves. Leaves are flattened, taping in to a long, acute point and with smooth edges which are crimped and pink sheathing at bases of tufts of leaves. The leaves size is ranges from 0.7 and 1.7 cm in width with average of 1 cm. The sympoidal leaf of *Acoruscalamus*are short heightedas compare to that of the vegetative leaves. The leaf has a single prominent mid vein and then on both sides slightly raised secondary veins and many, fine tertiary veins. The margin is curly edged or undulate(Chandera et al., 2014).

**Figure 4: Figure showing the leaves of *Acoruscalamus***

**Flowers**

This plant produces flowers very rarely in the months of march-May. Flowers are cylindrical in shape, ranges from 3-8cm in length, greenish brown in color and covered with the multitude of rounded spikes. The inflorescence of this plant consists of the two parts spathe which is leaf-like and spadix spike-like in appearance. The spadix arises from the middle of the spathe and densely covered with numerous green and yellow colored flowers which are present in diamond shaped pattern. Each flower have 6 tepals and 6 stamens. The flowers of this plant are small in size, sessile or permanently attached and densely packed in 5-10 cm of spadix. The spadix, at the time of expansion, can reach a length in between 4.9 and 8.9 cm. Blooming period starts from late spring to early summer depending on the latitude, grows wild in marshy places up to 2000 m altitude (Gagan et al., 2015).

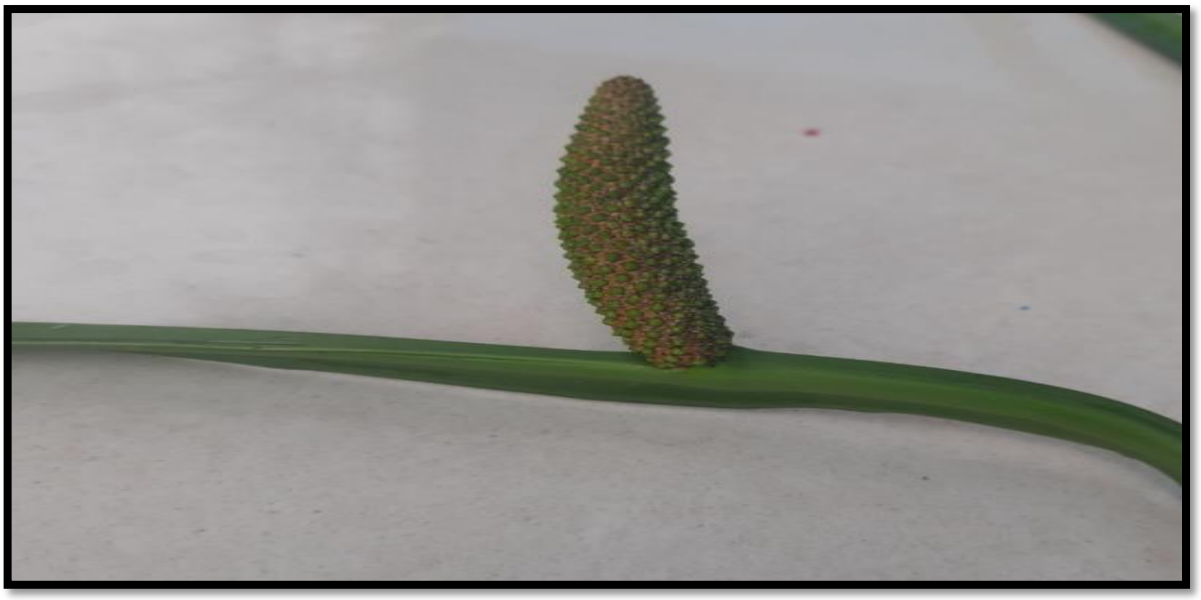


**Figure 5: Figure showing the flower of *Acoruscalamus***

**Fruits**

The fruits are small and berrylike and also containing few seeds. Flowering andFruiting occurs in July.Fruit has narrow, 6 to 14 in (15 - 35.6 cm) shiny leaves, thick and lush grass like in appearance. The leaves grow in two ranks, like opposing fans. Fruits of herb are generally flat, ranges from 0.5 in (1.3 cm) in width and tend to flop over. The insignificant flowers, little horns in appearance, are mainly produced in midsummer on erect hollow stems. Usually when grown in water produce flowers (Umamaheshwari et al., 2018).

**Chemical Constituents**

A wide range of phytoconstituents have been reported from Photochemical studies of *Acoruscalamus* include glycosides, alkaloids, flavonoids, saponins, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principle. The essential oil contains the major constituent like calamen, calameon, clamenolasarone and sesquiterpenes. Plant also contains a bitter glycoside named as acorine along with eugenol, pinene and camphene. The chemical constituents investigated from the various parts like rhizomes, leave and roots of the plant includes β- asarone, α- asarone, isoeugenol, cisisoelemicineelemicineand their methyl ethers, camphene, P-cymene, α-selinene, betagurjunene, aterpineol, terpinen-4-ol and a calacorene, acorone, acrenone, β-cadinene, camphor, acoragermacrone, 2-deca -4,7dienol, linalool, shyobunones and preisocalamendiol are also present. Acoradin, galangin, 2, 4, 5- trimethoxybenzaldehyde,

2, 5 dimethoxy benzoquinone, calamendiol, spathulenol and sitosterol have been isolated from *Acoruscalamus*(Chandera et al., 2017).

β-Asarone (isoasarone) is the major chemical constituent present in the plant. alpha-Asarone, elemicine, cis-isoelemicine, isoeugenol and their methyl ethers, camphene, P-cymene, beta-cadinene, camphor, beta-gurjunene, alpha-selinene, terpinen-4-ol, alpha-terpineol and acorone, acorenone, acoragermacrone, alpha-calacorene 2-deca-4,7-dienol,shyobunones, isohyobunones, calamusenone, linalool and pre-isocalamendiol are also present. And the other chemical constituents are Acoradin, galagin, 2,4,5-trimethoxy benzaldehyde, 2,5-dimethoxybenzoquinone, calamendiol, spathulenol and sitosterol have been isolated from *Acoruscalamus*(Kumar et al., 2013).

**Pharmacological Applications of *Acoruscalamus***

**Antibacterial Activity**

Haghighi et al., 2014investigated the anti-bacterial effect of rhizomes extract of *Acoruscalamus*against various bacterial strains such as *Staphylococcusaureus, Staphylococcus epidermidis*and *Escherichia coli* by using disc diffusion method to determine the susceptibility of bacteria at various concentrations ranges from (100-400 mg/ml). The Minimal InhibitoryConcentration (MIC) of rhizome extracts generally varied from 25-100 mg/ml against these susceptible bacteria.The ethanolic extracts plant showed maximum inhibitory activityhaving (16mm) diameter zone of inhibition against *Staphylococcus epidermidis.*The presence of compounds like phenyl propanoids, monoterpenes, sesquiterpenes and β-asarone in essential oil of the rhizome analysed by Gas Chromatography and Mass Spectrometry (GC-MS) techniques which showed the antibacterial properties. The various solvent extracts ofrhizome partcould be useful for effective formulation which helps in curing various kinds of infectious ailments.

**Antifungal Activity**

Rita et al., 2017examinethe presence of active compounds in the essential oil of rhizome part by using Gas Chromatography-Mass Spectroscopy (GCMS) and identify the antifungal effect of against *Candida albicans.* Agar well diffusion methodwere used to check the antifungal activity. Antifungal activity test results showed that the growth and biomass inhibition of *Candidaalbicans* increased with the increase of the oil concentration in wells. Results revealed that the MIC value of essential oil toward *Candidaalbicans* was 1% with the inhibition zone of 7.83 mm.

Rawal et al., 2015Evaluate antifungal activity of *Acoruscalamus* (rhizome) extracts of the plant. In experimental study the various solvents (methanol, ethanol, petroleum ether

and in aqueous) extractions tested against fungal pathogens *Fusariumoxysporum f. sp. lycopersici*by using paper disc diffusion method. Results showed that the acetone solvent extract of rhizome was most effective and at its 1000 mg/ml concentration showed the best antifungal activity with inhibition zone of 29.5mm against *Fusariumoxysporum f. sp. lycopersici.*

**Anti-inflammatory Activity**

Kim et al., 2008 investigated the anti-inflammatory activity of *Acoruscalamus*leaf (ACL) extract on human keratinocyte HaCaT cells. In this experimental studyHaCaT cells firstly treated with polyinosinic:polycytidylic acid (polyI:C) and peptidoglycan(PGN) and this treatment leads to the induction of the inflammatory reactions which were determined by using various methods like RT-PCR, immunofluorescence staining ELISA assay, immunoblotting. HaCaT cells induced for the production of pro inflammatory cytokines, interleukin-8 (IL-8) and/or interleukin-6(IL-6) expressions after treatment with immunostimulant. ACL inhibited the expression of IL-8 and IL-6 RNAand protein levels, and attenuated the activation of NF-$\kappa\beta$ and IRF3 after polyI:C treatment. ACL alsoinhibited expression of IL-8 and activation of NF-κβ following PGN induction. These results concluded that ACL inhibits the production of pro-inflammatory cytokines throughvarious mechanisms which are responsible for inflammation and therefore plant may be serves as efficacious anti-inflammatory agent for curing various types of skinailments.

**Anti-oxidant Acivity**

Chaubey et al., 2017successfully reported the antioxidant activities of methanolic extracts of rhizome part of *Acoruscalamus*. The antioxidant activity of plant methanolic extracts were examined by DPPH radical scavenging activity, reductive ability and metal chelating activity. The maximum reducing power observed in sample 15R which was collected from the site Gumti of district Almora, Uttarakhand (IC50 = 104.62 ± 0.922 µg/mL) while 20R which was collected from the site Khedagao of district Almora, Uttarakhand (IC50 = 241.81 ± 0.762 µg/mL) exhibit lowest reducing power activity. Maximum antioxidant activity (DPPH assay) was observed in 7R sample collected from site chaukhutiya of district Almora (IC50 = 143.35 ± 0.741 µg/mL) while 17R from site Donprewa of district Nainital exhibited lowest radical scavenging activity (IC50 = 919.55 ± 0.588 µg/mL) and maximum antioxidant activity (metal chelating) was observed in 20R (IC50= 308.57 ± 0.855 µg/mL) while 6R sample collected from site Paithani (IC50= 1439.35 ± 2.896 µg/mL) showed minimum chelating activity. Finally, it

has been concluded that methanolic extracts of *Acoruscalamus*serves as a best source of natural antioxidant for medicinal uses.

**Anti-Diabetic Activity**

Prisilla et al., 2012reported the male albino rats were rendered diabetic by oral administration of STZ (40 mg/kg, intraperitoneally). In this experimental study *Acoruscalamus* rhizome extract (200 mg/kg) was administered orally to diabetic rats upto 21 days to know the antihyperglycemic activity. Results showed that oral administration of methanol extract of rhizome part of *Acoruscalamus*in streptozotocin (STZ) induced diabetic rats showed significant restoration of the levels of blood and glucose level. Male albino rats observed after treatment extract results shows that blood glucose, lipid profile (total cholesterol, LDL and HDL- Cholestrol), glucose 6-phosphatase, fructose 1,6 bis phosphatase levels and hepatic marker enzymes (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase) were decreases when compare with diabetic control. Plasma insulin, tissue glycogen, glucose- 6-phosphate dehydrogenase levels were increased significantly compared to diabetic control. Results from the study revealed that the methanolic extracts of rhizome part possess the effective antihyperglycemic activities and considered as most powerful drug for treatment of diabetes.

**Anti-Cancerous Activity**

Haghighi et al., 2017tested the anti-cancer activity of the ethanolic and methanolic extracts of *Acoruscalamus* (rhizome) and essential oil of the rhizome on human gastric cancer cell line (AGS). The study showed that anticancerous activity of extract and essential oil inhibited the growth of AGS cells and also inhibited the angiogenesis in HUVEC cells. Results of the study showed that the extracts and the essential oil of *Acoruscalamus*caused G1 arrest in AGS cells and downregulation of Oct4 and NS after extract treatment. The GC-MS analysisresults also exhibited the presence of new compounds such as epiprezizaene, valencene and isocyclocitral in essential oil of *Acoruscalamus.*

**Insecticidal Activity**

Kumar et al., 2015evaluated thelethal concentration 50 (LC50) values of ethanolic extract of *Acoruscalamus* (rhizome) against larvae, adult males and females of *Drosophila melanogaster* were recorded as 109.54, 52.51 and 41.11 mg/L respectively. Genotoxicity of adult's flies were determined at 30 and 55 mg/L ethanolic extract of *Acoruscalamus*(rhizome). The mean comet tail length was 4.24±0.653 µm and

6.13±0.721 μm and the respective DNA damage was 5.1% and 7.3% with reference to controls. Results showed that ethanolic extract of *Acoruscalamus* rhizome showed higher effect as insecticidal activity in adults than larvae of *Drosophila melanogaster*. But the results from genotoxicity studies further support the insecticidal activity against adult stages of *Drosophila melanogaster*. The study reflects ethanolic extract of *Acoruscalamus* could be used as an alternative pest control negotiator for minimizing the noxious effects of pesticides in the environment.

**Antispasmodic Activity**

Gilani et al., 2006 reported the antispasmodic and anti-diarrhoeal effect of *Acoruscalamus* (rhizome). In this experiment the crude extract of rhizome part firstly tested positive for the presence of phytochemicals alkaloid, saponins and tannins. The crude extract of *Acoruscalamus* (rhizome)was tested inthe isolated rabbit jejunum which caused inhibition of spontaneous and high $K^+$(80 mM)-which is responsible to produce contractions, with respective $EC_{50}$ values of 0.42 ± 0.06 and 0.13 ± 0.04 mg/mL (mean ± SEM; $n$ = 6–8), thus showing spasmolytic activity, mediated possibly through calcium channel blockade (CCB). The CCB activity was confirmed when pre-treatment of the tissue with crude extract (0.3 –1.0 mg/mL) caused a rightward shift in the $Ca^{++}$ dose-response curves regarding this similar effects to that also caused by verapamil, a standard calcium channel blocker. These results suggest that this plant serves as potential agent as an intestinal relaxant showing spasmolytic effect that mediated through the presence of CCB-like constituent(s) which is concentrated in the n-hexane fraction and this study provides a strong mechanistic base for its traditional use in treatment of various gastrointestinal disorders such as colic pain and diarrhea.

**Analgesic and Anticonvulsant Activity**

Jayaraman et al., 2010 evaluated the analgesic effects of methanolic roots extracts by using two methods acetic acid induced Writhing response and Rat caudal immersion method. While the anticonvulsant effect were analyzed by utilizing pentylenetetrazol induced convulsion methods. Administeration of the roots extracts oral doses at the concentration of 100 and 200 mg/kg, showed the protective effect against the pain models in mice. MEAC significantly increased the latency period in seizures induced by pentylenetetrazole (PTZ) in mice.

Kaushik et al., 2018 reported the anticonvulsant activity of ethanolic roots extract of by maximal electroshock seizure (MES) and pentylenetetrazol (PTZ)-induced seizure models on albino (Wistar strain) rats. Result of study showed that extract treatment

reduced the duration of tonic hind limb extension in MES model, and there was increase in latency and occurrence of convulsions in PTZ model.

**Effect on Neurological Activity**

Lee et al., 2014evaluated the anxiolytic or anti-anxiety activity of major phytocompound α -asaroneisolated from *Acorusgramineus*in rat model used in experimental study in which the anxiety behavior induced due to repeated oral administration stress hormone corticosterone (CORT). The anti-anxiety activity of compound in ratswas evaluated by the elevated plus maze (EPM) test and the hole-board test (HBT) tests of anxiety. The results of the study give the detailed information of the neurobiological mechanisms in rat models responsible for changes in emotions induced by repeated administration of high doses of corticosterone hormone or by elevated levels of hormones associated with chronic stress. The α-asarone (AAS) compound from *Acorusgramineus* did exhibit anxiolytic-like effects in animal models of anxiety.

## RESEARCH GAP IDENTIFICATION

Review of literature shows that the Plant (*Acoruscalamus*) possesses various pharmacological applications which are useful for the treatment of various types of diseases. To explore some other beneficial properties of *Acoruscalamus* we need to work on the following aspects.

- *In silico* Prediction of antimicrobial activity of major phytocompounds of *Acoruscalamus*with*Pseudomonas aeruginosa* penicillin-binding protein 3 (PDB ID 6VJE) and Cytochrome P450 14 alpha-sterol Demethylase target proteins.
- ADMET screening of major phytocompounds of *Acoruscalamus* to determine the absorption, toxicity, and drug-likeness properties of phytocompounds.
- Exploring the synergistic potential of the extract of *Acoruscalamus* and its bioassay guided subfractions against resistant microbial strains.
- Assessment of antimicrobial effect of the cow urine plant extract consortium against the resistant microbial strains to overcome antimicrobial resistance.

## OBJECTIVES

### Objective 1

a. Collection and Identification of Medicinal plant.
b. Preparation of extracts and bioassay guided fractionation of *Acoruscalamus*.

### Objective 2

a. Assessment of antimicrobial activity of prepared extracts of *Acoruscalamus* and its bioassay guided subfractions.

b. Assessment of synergistic activity of prepared extracts and its subfraction in combined with antibiotic against Bacterial strains.

## Objective 3

*a. In silico* Prediction of antimicrobial activity of major phytocompounds of *Acoruscalamus.*

b. Toxicity Prediction of Major Phytocompounds of *Acoruscalamus*

## Objective 4

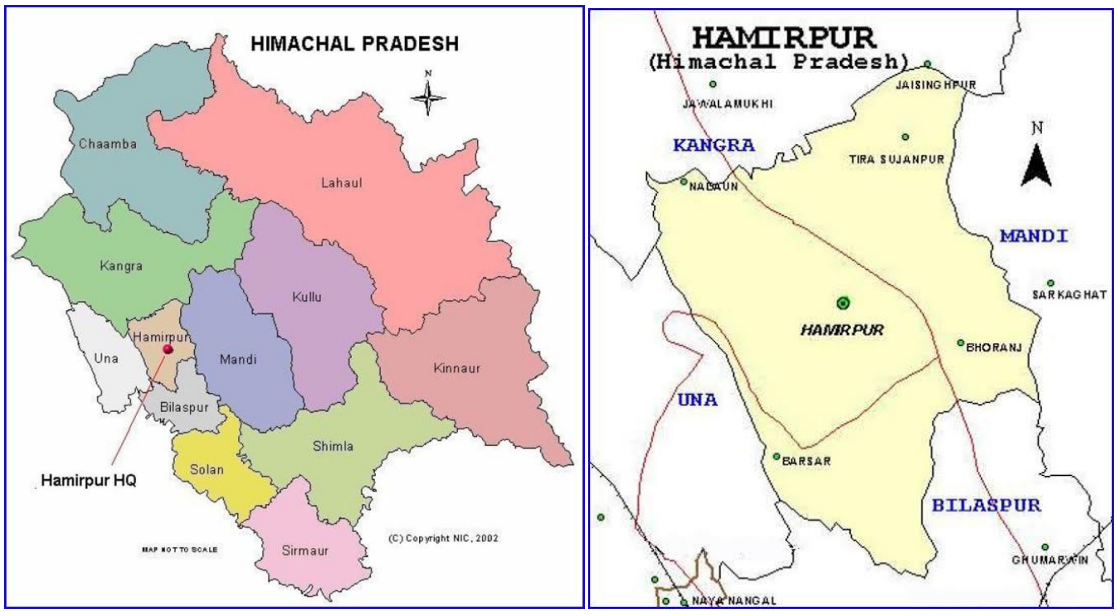
a. Quantification of active Phytocompounds present in the selected extracts of *Acoruscalamus* and its subfractions by HPTLC.

## PROPOSED METHODOLOGY

## Objective 1

### Collection and Identification of Plant

Medicinal plant used in the study will be collected from District Hamirpur, H.P. and identified from the Department of Biosciences, Himachal Pradesh University, Shimla. The collected plants (leaves) will be washed thoroughly under the tap water and followed with 70% of ethanol to eliminate the dust and other foreign particles (Minocheherhomji et al., 2014).




https://hphamirpur.nic.in/map-of-district/

**Figure 6: Figure showing the map of plant material collection site**

**Preparation of extracts of rhizome and leaves parts of *Acoruscalamus***

The rhizome and leaves of *Acoruscalamus*will be collected from the Hamirpur district of Himachal Pradesh and thoroughly washed with running tap and then dried in hot air oven at 35-40ºC and ground to fine powder. The extracts of plant will be prepared by using a Soxhlet extraction method. After that the prepared extracts dissolved in autoclaved distilled water and successively fractionated with solvents such as ethanol, methanol, cowurineand aqueous. The Prepared extracts and its active subfractionsi.e. chloroform,ethyl acetate and n-Butanolof rhizome and leaves of *Acoruscalamus*will be screened for various phytoconstituents such as phenolics, tannins, flavonoids, phytosteroids and saponins as per standard methods (Chandel et al., 2019).

**Procedure:-**

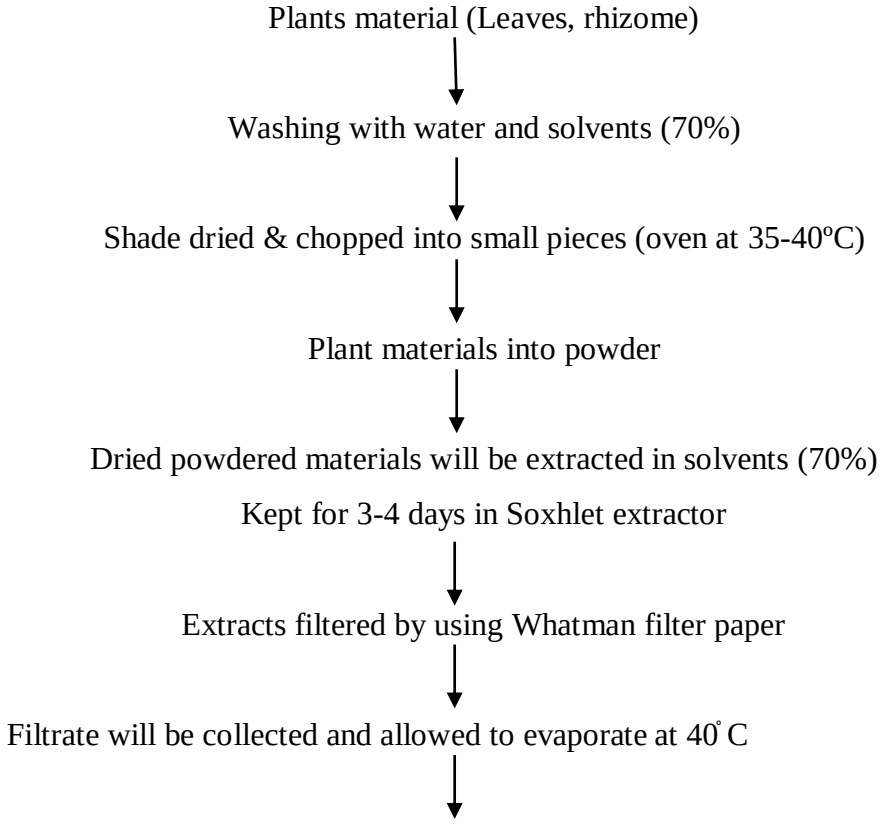

Plants material (Leaves, rhizome)
Washing with water and solvents (70%)
Shade dried & chopped into small pieces (oven at 35-40ºC)
Plant materials into powder
Dried powdered materials will be extracted in solvents (70%)
Kept for 3-4 days in Soxhlet extractor
Extracts filtered by using Whatman filter paper
Filtrate will be collected and allowed to evaporate at 40° C
Dried crude extracts stored in air tight dark bottles at 4° C

**Figure 7: Flowchart of procedure of Extract Preparation**

## Objective 2

**Assessment of antimicrobial and synergistic activity of prepared extract of *Acoruscalamus* and its subfractions.**

**Fungal and Bacterial Cultures**

Standard fungal and bacterial cultures of the microbial strains will be procured from the sourced from IMTECH, Chandighar. Selected pathogenic strains of microbes:

- *Staphylococcus aureus*(MTCC 737 )
- *Pseudomonas aeruginosa*(MTCC 1688)
- *Escherichia coli* (MTCC 739)
- *Bacillus cereus* (MTCC 441)
- *Salmonella enterica ser. Paratyphi*(MTCC 735)
- *Acinetobacter baumanii*(MTCC 1425)
- *Candida albican*(MTCC 183)
- *Candida aurigiensis*(MTCC 1664)

**Assessment of antimicrobial and synergistic activity**

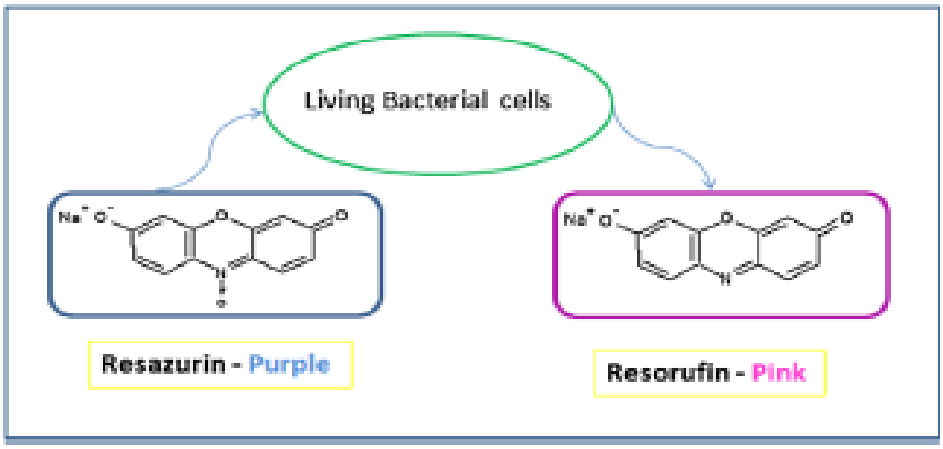
The antimicrobial and Synergistic effect of medicinal plant on microbial strains will be screened by using the agar-well diffusion method (Guleria et al., 2020).

**Procedure:-**

- The freshly prepared sterile media usually Muller Hinton agar mediafor the growth of bacterial strains and Sabouraud Dextrose Agar for the growth fungal strains will be poured (20 ml) into sterile petriplates and allowed to solidify under aseptic conditions.
- An inoculum suspension (20 µl) will be spread on selective plate of media and labelled properly.
- 5 or 6 wells of 6mm diameter will be made in the agar media by using sterile cork borer in the solidified medium in petriplates.
- Different volumes of each plant extract (5 µl-40 µl) will be added to respective well
- Seeded medium then allowed to stand for diffusion and after that incubated at 37$^{o}$C for bacteria and at 30$^{o}$C for fungi for 24hr.
- All extracts will be tested against all the microbial strains.
- Antimicrobial activity of each plant extract will be determined in terms of diameter of zone of inhibition around each well by each extract at the end of incubation period.
- The inhibitory zone indicates the absence of bacterial growth around the wells and it will be reported as positive and sensitive towards that extract.
- Zone diameter of inhibition will be measured by using diameter measurement scale. Zone of inhibition (ZOI) = Total diameter of well along with zone of inhibition – diameter of well.

**Minimum inhibitory concentration (MIC) against selected microbial strains**

Dilution method will be used to measure the Minimum inhibitory concentration of antimicrobial agents. In this method ability of the microbes are tested to produce visible growth in the wells microtitre plate which containing the various dilutions of the antimicrobial agent. MIC is defined as the minimum concentration of an antimicrobial agent that required inhibiting the visible growth of tested microbial isolates (Turnidgeet al., 2013). Resarzurin dye is fluorogenicoxidation-reduction indicator which mainly used to determine the amount of aerobic respiration. The blue color of dye changes to pinkcolor and give fluorescent when enzymesoxidoreductases present in viable cells showreaction in solution reduce the dye to resorufin product (Fig.8). Resazurin is again reduced to hydroresorufin which is colourless and non-fluorescent (McNicholl., 2007).




(https://www.google.com/url?sa=i&url=https%3A%2F%2Fwww.researchgate.net)

**Figure 8: Active living cell cause reduction of resazurin (purple-blue) to resorufin (pink-colorless).**

- For antimicrobial activity microtitre plates (Tarson) under aseptic conditions in laminar air chamber will be used for antimicrobial assay.
- Firstly the first row of microtitre plate will be filled with 100 µl of test materials which is prepared in 10% (v/v) DMSO or either in sterile water.
- Wells of plate will be filled with 100 µl of NB. Two fold serial dilution (throughout the column) will be achieved by starting transferring 100 µl test material from first row to the subsequent wells in the next row of the same column and so that each well had 100 µl of test material in serially descending concentrations.
- Resazurin solution (10 µl)will be added in every well of microtitreplate. Finally, 10 µl quantity of bacterial suspension will be taken.

- For prevention of the bacterial culture from dehydration, each plate will be wrapped loosely with cling film.
- Two sets of controls will be taken during this method which includes: (a) a column with in which antibiotic is added taken as positive control while with all solutions with the excluding of the test extract taken as negative.
- The plates will be incubated in BOD incubator at 37° C for 24 h after incubation color change in color of dye will be observed visually. When color change from purple to pink will be taken as positive.
- The minimum amount of extract at which the color change in dye occurred will be denoted as the MIC value for the particular test.

## Objective 3

***In silico* Prediction of antimicrobial activity of major phytocompounds of*Acoruscalamus.***

**Bioinformatics tools:** Several bioinformatics techniques will be used in this research. AutoDockVina (Trott and Olson, 2010) Open Babel GUI (O'Boyle et al., 2011),Molispiration(https://www.molinspiration.com/),admetSAR(http://lmmd.ecust.edu.cn/admetsar1/predict), PROTOX-II (http://tox.charite.de/protox_II/) and Chimera 1.8.1 (Pettersen et al., 2004).

**Retrieval and preparation of target protein**

Crystal structures of *Pseudomonas aeruginosa* penicillin-binding protein 3 having PDB ID 6VJE (Kumar et al., 2020) and Cytochrome P450 14 alpha-sterol Demethylasehaving PDB ID: 1EA1 (Podust et al., 2011)will be retrieved from the protein data bank (https://www.rcsb.org/) (fig. 1). The protein structure will be generated using the Auto-Dock tool 1.5.4. The chosen structure will be processed to add missing hydrogens to the raw structure, create disulfide bonds, and assign proper bond orders.

**Ligand preparation**

Three dimensional structures of major phytocompounds (β asarone, Elemicine, Eugenol, Methyl isoeugenol, Camphene, P-cymene, α-selinene, β-gurjunene, β-cadinene, Acorone and Shyobunone) of *Acoruscalamus.*Plant and antibiotics penicillin and fluconazole will be derived from Pubchem server (www.pubchem.com) in .sdf format. The .sdf file of phytoconstituents will be changed into PDB format with the help of Open Babel tool (Wang et al., 2009).

**Molecular docking of phytocompounds *Acoruscalamus***

Phytocompounds and antibiotics (β-asarone, elemicine, eugenol, methyl isoeugenol, camphene, P-cymene, α-selinene, β-gurjunene, β-cadinene, acorone, shyobunone, penicillin and fluconazole) will be used to perform molecular docking studies against bacterial (6VJE) and fungal (1EA1) proteins to know the potential drug to inhibit proteins implicated in drug resistance. In the current research molecular docking will be performed by using AutoDockVina software (Trott and Olson., 2010).

**Toxicity Prediction of Major Phytocompounds of *Acoruscalamus***

ADMET screening will be done to determine major properties of phytoconstituents like the absorption of drug, toxic effect, and drug-likeness property. The 3 dimentional structures of phytocompounds (β-asarone, elemicine, eugenol, methyl isoeugenol, camphene, P-cymene, α-selinene, β-gurjunene, β-cadinene, acorone and shyobunone) will be saved and file should be in .smiles format and the saved format uploaded SWISS ADME and PROTOX-II webservers (Charite University of Medicine, Institute for Physiology, Structural Bioinformatics Group, Berlin, Germany) (Banerjee et al., 2018; Rolta et al., 2020a,b,c; Salaria et al., 2020).

## Objective 4

**Quantification of active Phytocompounds present in the selected extracts of *Acoruscalamus* and its subfraction by HPTLC.**

Analysis was performed using *Series 200 Ic pump* (Perkin Elmer) equipped with Reverse phase Lichrosorb C18-5 µm (4 × 125 mm) column (Merck) and *785 A programmable Absorbance Detector* (Applied Biosystem) using an HPLC column equipped with a Waters pump system. Samples were dissolved in methanol and a volume of 5 µL was injected into HPLC(Zhao et al., 2010).

# THESIS OUTLINE

| | | |
|---|---|---|
| **Chapter 1** | **Introduction** | This chapter provides the background on the research topic. |
| **Chapter 2** | **Literature Review, Research Gap Identification and Objectives** | This chapter will be summarized the major studies that have been published on a research topic and methodologies used by different workers.<br>Point out the principal deficiencies of these studies and explaining how your research proposes contributes to the literature. |
| **Chapter 3** | **Methodology** | This chapter provides the details of all the methods, procedure and techniques used to identify information about the present research. |
| **Chapter 4** | **Result and Discussion** | In this chapter results of present research work shown and discussed |
| **Chapter 5** | **Summary and Conclusion** | This chapter will summarized the result findings and give the scopes for further research. |
| **Chapter 6** | **References** | References includes the detailed information will be written in Harvardreference system. |

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# CERTIFICATE
## (SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Synopsis for Ph.D. Thesis titled, **"Evaluation of *In vitro* and *In silico* antimicrobial and synergistic potential of *Acorus calamus* collected from Himachal Pradesh, India"**, submitted by **Mrs. Kumari Shalini** to the Career Point University, Hamirpur (H.P.), India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in the Division of Microbiology, Department of Pharmacy, School of Pharmaceutical and Health Sciences has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated **16-03-2021.**

Dr Shikha Kumari
**(Supervisor)**
Assistant Professor, Microbiology
Division Microbiology, Department of Pharmacy

**Research Advisory Committee**

Dr Hem Chander
**(Member)**
Assistant Professor, Botany

Dr Younis Ahmad Hajam
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Assistant Professor, Zoology

Dr Vijay Kumar
**(Member)**
**(HOD)** Division of Microbiology

20/4/2021
Dr Yogesh Kumar Walia
**(Associate Dean R & D)**